Korean J. Chem. Eng., **29**(7), 925-930 (2012)
DOI: 10.1007/s11814-011-0272-5

Production of cellulases and β-glucosidase in *Trichoderma reesei* mutated by proton beam irradiation

You Ree Jung*, Hyun Yong Shin*, Hah Young Yoo*, Youngsoon Um, and Seung Wook Kim*,†**

*Department of Chemical and Biological Engineering, Korea University, 1, Anam-dong, Sungbuk-gu, Seoul 136-701, Korea
**Center for Environmental Technology Research, Korea Institute of Science and Technology, Seoul 136-130, Korea
(*Received 23 June 2011 • accepted 24 October 2011*)

Abstract−To obtain mutant strains producing high levels of cellulases (FPase and CMCase) and β-glucosidase, *Trichoderma reesei* KCTC 6950 was mutated by proton beam irradiation. Five mutants were selected out of 1,000 mutants of *T.reesei* treated with proton beam irradiation, based on their ability for enzyme production on a plate screening medium. In submerged cultures containing Mandel's fermentation medium, the mutant strain T-2 (MT-2) demonstrated a 165% increase in the activity of FPase, a 146% increase in the activity of CMCase, and a 313% increase in the activity of β-glucosidase, compared with the wild type strain. Additionally, the properties of high level β-glucosidase produced by MT-2 were the same as those of the wild type strain, e.g., an optimum pH of 4.8, and an optimum temperature of 65 °C. Moreover, the protein concentrations of β-glucosidase produced by the wild type strain and MT-2 were measured by SDS-PAGE, and then β-glucosidase activities were detected by the MUG-zymogram assay.

Key words: Cellulases, β-Glucosidase, *Trichoderma reesei*, Proton Beam Irradiation, Mutation

INTRODUCTION

Bioconversion of lignocellulosic biomass, such as agricultural wastes and woody biomass, to ethanol has been extensively studied in the field of bioenergy [1]. When lignocellulosic biomass is converted to ethanol, the cost of enzymes accounts for a large portion of the entire project cost [2,3]. The term "cellulase" broadly refers to enzymes that catalyze the hydrolysis of the β-1,4-glucosidic bonds joining individual glucose units in the cellulose polymer [4]. Cellulase production is one of the important processes for the successful enzymatic conversion of cellulosic biomass to ethanol [5,6]. The cellulase complex secreted by filamentous fungi consists of three major enzyme components, endo-1,4-β-glucanases (EC 3.2.1.4), 1,4-β-D-cellobihydrolases (EC 3.2.1.91), and 1,4-β-glusosidases (EC 3.2.1.21), which act synergistically during the conversion of cellulose to glucose [7,8]. Various endo-1,4-β-glucanases act randomly along the cellulose chains, thus producing cellulose fragments. 1,4-β-D-cellobihydrolase, which acts as an exoglucanase, releases cellobiose as the main product; then, 1,4-β-glusosidase hydrolyzes cellobiose to glucose [9].

Among the filamentous fungi, *Trichoderma* species have been especially famous for producing cellulolytic enzymes with relatively high enzymatic activity [10,11]. However, they showed very low levels of β-glucosidase activity, which results in decreased hydrolysis rates of glucose from cellulose, due to product inhibition of the endo-1,4-β-glucanases and 1,4-β-D-cellobihydrolases by cellobiose accumulation [12,13]. To overcome the problems of β-glucosidase being rate limiting in the production of glucose from cellulose when using cellulase produced by a filamentous fungi, many researchers have reported that *T. reesei* was transformed by mutation to produce cellulolytic enzymes with higher specific activity and greater efficiency [14]. Recently, proton beam irradiation has emerged as a new mutation method due to its characteristic high local energy, comparable to beams of X-rays, gamma-rays and electrons [15,16]. To improve the production of both the cellulases and β-glucosidase by *T. reesei*, mutants were obtained by proton beam irradiation.

In this study, *T. reesei* KCTC 6950 was mutated by proton beam irradiation to obtain improved strains capable of producing high levels of cellulases and β-glucosidase for efficient saccharification. The mutant strains were investigated for enzyme production, clear hydrolysis zone, and growth rate. In addition, the pH and temperature properties of β-glucosidase produced by the wild type strain and MT-2 were characterized. Moreover, the amount of β-glucosidase excreted by the wild type strain and MT-2 was measured by SDS-PAGE, and then detected by the MUG-zymogram assay.

MATERIALS AND METHODS

1. Microorganisms

The wild type strain used as the control was *Trichodema reesei* 6950 obtained from the Biological Resource Center/KCTC (Seoul, Korea). The fungus was maintained on potato dextrose agar (PDA) plates at 28 °C for 7-10 days, and stored at 4 °C when spores were formed.

2. Mutagenesis and Plate Screening Methodology

The proton beam (MC-50 Cyclotron) was irradiated at the Korea Cancer Center Hospital (KCCH, Seoul, Korea). For proton beam-mutagenesis, the spores from a one-week-old PDA slant were suspended in 30 ml of sterile water containing 0.2% Tween 80. The spore concentration was determined by counting with a Bürker counting chamber, and a suspension containing 5×10^8 spores/ml was treated by proton beam irradiation. The spore suspensions were exposed to proton beam irradiation (doses of 0.3- 3.0-kGy doses) at a beam

†To whom correspondence should be addressed.
E-mail: kimsw@korea.ac.kr

energy of 45 MeV, and a spore survival rate of 0.01% was later determined. Surviving spores were incubated at 30 °C for three days on the plate-screening medium. The plate-screening medium for selection of hypercellulolytic mutants contained Mandel's mineral salts solution, with the addition of 0.2% carboxymethylcellulose (CMC) sodium salt, 1.7% agar, 0.1% Triton X-100, and 0.4% L-sorbose as colony restrictors [17]. Hypercellulolytic mutants were selected using Gram's iodine solution on the basis of clearing zones around the cellulase-producing colonies (clearing zone ratio: clearing zone diameter/colony diameter) [18]. These mutant strains were transferred to PDA plate agar media and later used for determining their cellulase production abilities.

3. Culture Conditions and Enzyme Production

The production of enzymes was induced in submerged cultures of the wild type and mutant strains. A 10 mm agar plug taken from a seven-day-old PDA culture was inoculated into a 250 ml Erlenmeyer flasks containing 100 ml inoculum medium [19]. The initial pH was adjusted to 5.5 by addition of 10% (v/v) H_2SO_4. Inoculum was incubated at 30 °C and 150 rpm for three days, and then used to inoculate the fermentation media. For fermentation preparation, 10 ml of inoculum was used to initiate growth in 250 ml Erlenmeyer flasks containing 100 ml Mandel's fermentation medium. This medium contained Mandel's mineral salts solution with the addition 10 g L^{-1} microcrystalline cellulose as a carbon source, and the pH was adjusted to 4.8. Enzyme production was continued for seven days at 30 °C, and samples were centrifuged (13,000 g, for 10 min at 4 °C) to remove solids. The supernatants were assessed for cellulase activity and protein concentration.

4. Enzyme Assays

Cellulases (FPase and CMCase) and β-glucosidase activities were determined in the supernatant samples collected after one to seven days culture. The filter paper (FPase) activity was measured as international units (IU). It was assayed by a modification of the method [20], as follows: 1.0 ml of 0.05 M citrate buffer (pH 4.8) was added to test tubes containing 1.0 ml of diluted enzyme solution and 50 mg of filter paper (Whatman No. 1. 1×6 cm). The reaction mixture was incubated at 50 °C for 60 min and 3 ml of dinitrosalicylic acid (DNS) reagent was added to stop the reaction. The tubes were transferred to 100 °C boiling water for 5 min, followed by addition of 10 ml of distilled water. The contents of the tubes were mixed and the color formed was read in a spectrophotometer at 575 nm. The endoglucanase (CMCase) activity was determined using CMC as the substrate, as follows: 1.0 ml of 1.0% CMC solution was incubated with 0.5 ml of diluted enzyme solution, diluted with 0.05 M citrate buffer (pH 4.8) at 50 °C for 30 min. Then, 3 ml of DNS reagent was added and the solution was boiled at 100 °C for 5 min. Finally, 10 ml distilled water was added and the absorbance was measured at 575 nm. The β-glucosidase activity was assayed in 1 ml of a reaction mixture containing 0.1 ml of diluted enzyme solution and 0.9 ml of 1 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) in 0.05 M citrate buffer (pH 4.8) at 50 °C for 30 min. After the incubation, 1 ml of 1 M Na_2CO_3 solution was added to the mixture and allowed to develop color. Then, 10 ml of distilled water was added and the release of *p*-nitrophenol was determined at 400 nm. At all dosages, 1 unit of activity was the amount of enzyme required to release 1 μmol glucose (DNS procedure) or *p*-nitrophenol per min under the conditions of the assay. Protein concentration was measured by the Bradford method with bovine serum albumin (BSA) as a standard [21].

5. Characteristics of β-Glucosidase

The optimum pH was evaluated by measuring the β-glucosidase activities of wild type and mutant strain at the optimum culture time (five days) with *p*NPG as the substrate at 50 °C and various pHs. The buffers used were 0.05 M citrate buffer (pH 3.0-6.0), and phosphate buffer (pH 6.0-8.0). The optimum temperature was evaluated by measuring the β-glucosidase activities of the wild type and mutant strain at the optimum culture time and pH, at different temperatures (30-80 °C), with *p*NPG as the substrate. The thermotolerance of β-glucosidase was determined at the optimum culture time, pH, and temperature with *p*NPG as the substrate, after incubating the enzymes at different temperatures (30-80 °C) for both 30 and 60 min.

6. Protein Electrophoresis and Zymogram Assay

The crude enzyme preparation was concentrated by a centrifugal vacuum concentrator using the supernatant from a culture grown for five days. The crude enzyme was examined by SDS-PAGE as previously described [22]. For zymogram analysis, the samples were mixed with Laemmli's sample buffer and analyzed using an 8% (w/v) polyacrylamide gel without heat denaturation. Following SDS-PAGE, the gel was immersed in a solution containing 1 mM 4-methylumbelliferyl-β-D-glucoside (MUG), 0.05 M citrate buffer (pH 4.8), and 1% Triton X-100 solution for 30 min at 50 °C [23,24]. Enzyme activity within the gels was detected by the appearance of fluorescent bands that were visualized under shortwave UV light illumination, and photographs were acquired using an imaging GEL DOC system (Bio-Rad, U.K).

RESULTS AND DISCUSSION

1. Mutagenesis of *Trichoderma reesei* by Proton Beam Irradiation

The wild type *T. reesei* spores were exposed to 0.3 to 3.0 kGy doses of proton beam irradiation at a beam energy of 45 MeV. After irradiation treatment, the survival curve demonstrated a 0.01% survival rate of *T. reesei* spores at a dose of 1.5 kGy (Fig. 1). Initially, the survival rate decreased slightly with increased proton beam irradiation starting from a dose of 0.3 kGy, but then sharply decreased when

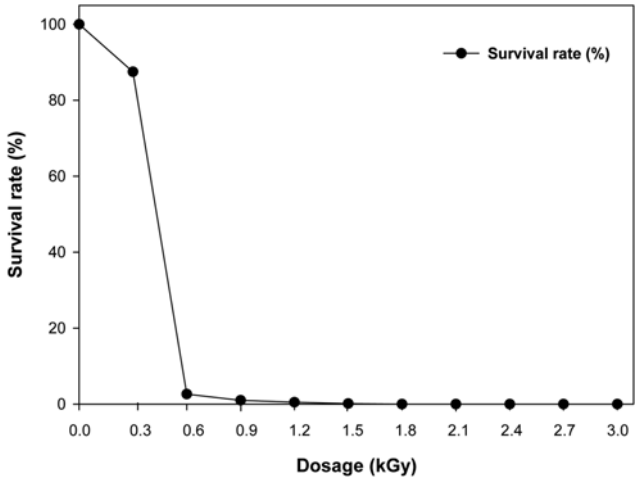

Fig. 1. Survival rate curve of *Trichoderma reesei* after proton beam irradiation.

the dose was increased from 0.3 to 0.6 kGy. Later, the survival rate was decreased as the dose increased. It was reported that the degree of mutagenesis is a critical factor, affecting how small a dose of radiation will be needed to increase the rate of mutagenesis and how large a dose will cause cell death. Small mutant dosage will not sufficiently increase the frequency of mutants, but over dosage of mutant will cause cell death [25]. Consequently, the suitable dose for proton beam irradiation was found to be 1.5 kGy, and the 0.01% surviving spores were spread on the plate-screening medium for selection of hypercellulolytic mutants producing three major enzymes (FPase, CMCase, and β-glucosidase).

2. Enzyme Activity of *T. reesei* Mutants

After mutagenesis, qualitative screening of mutants was conducted based on the diameter of the clearing zone surrounding the colony on the plate-screening medium. Five mutants were isolated based on enzyme production ability on the plate-screening medium, and were designated as mutant strains T-1 to T-5 (MT-1 to MT-5). The wild type and mutant strains were cultured in shake flasks containing Mandel's fermentation medium at 30 °C for five days. As shown in Table 1, in spite of the qualitative screening diameter of the clearing zone on the plate-screening medium, the two mutants (MT-3 and MT-5) had low cellulase (FPase and CMCase) activities in comparison to the differences found in the high activity of the β-glucosidase compared to the wild type strain. By contrast, the mutant strain MT-1 and MT-4 produced high activities of the cellulases (FPase and CMCase), but β-glucosidase activities were decreased, compared with wild type strain. Interestingly, this result shows the influence of the β-glucosidase, in that the clearing zone ratio of the two mutants (MT-3 and MT-5) having high level production of β-glucosidase were enhanced more than the MT-1 and MT-4 having low level production of β-glucosidase. These results suggest that the production of significantly increased levels of β-glucosidase was insensitive to catabolite repression by glucose in the cellulolytic system [26]. Particularly, MT-2 showed a ~165% increased activity of FPase (0.61 IU ml^{-1}), 146% increased activity of CMCase (2.74 IU ml^{-1}), and 313% increased activity of β-glucosidase (0.25 IU ml^{-1}) compared to the wild type strain. Also, the ratio of hydrolysis halo diameter and growth rate by MT-2 was enhanced more than that of the wild type strain (Fig. 2). Therefore, MT-2 was selected because of its enhanced enzyme production, clear hydrolysis zone, and rapid growth on the plate-screening medium.

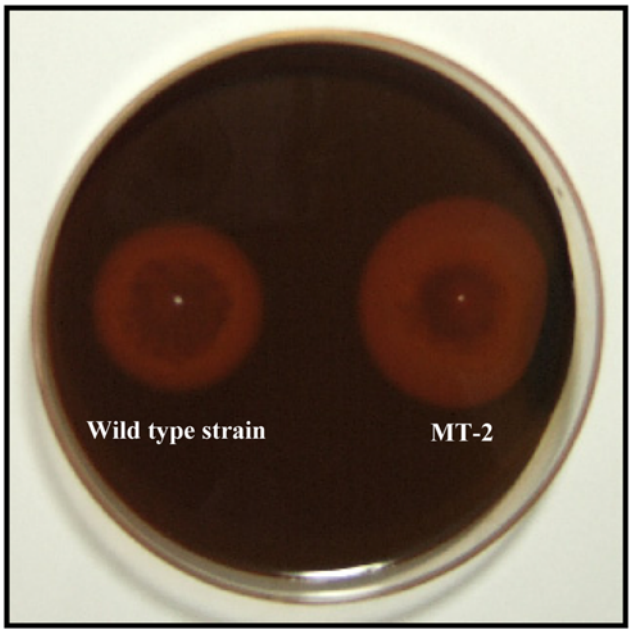

Fig. 2. Clearance zones of wild type and mutant strain T-2 (MT-2) on the plate-screening medium. The plate was incubated at 30 °C for 3 days, and then stained using Gram's iodine solution.

2-1. Time Course of Enzyme Production by Mutant Strain T-2 (MT-2)

The MT-2 demonstrated increased activities of cellulases (FPase and CMCase) and β-glucosidase at all intervals of culture time, as compared with the wild type strain (Figs. 3(a), (b), and (c)). It is evident that the highest activities of enzyme were obtained after five days of fermentation. Both cellulases and β-glucosidase activity were significantly decreased after six days. It may be attributed

Table 1. Comparison of enzyme production from wild type and mutant strains

Strains	Clearing zone ratio	FPase		CMCase		β-Glucosidase		Protein conc. (μg ml^{-1})
		Enzyme activities (IU ml^{-1})	Increased activity (%)	Enzyme activities (IU ml^{-1})	Increased activity (%)	Enzyme activities (IU ml^{-1})	Increased activity (%)	
Wild type strain	1.35	0.37±0.03	100	1.88±0.05	100	0.08±0.01	100	3.86±0.05
MT-1	1.36	0.38±0.04	102	1.90±0.04	101	0.07±0.03	-	ND
MT-2	1.74	0.61±0.05	165	2.74±0.02	146	0.25±0.02	313	4.69±0.02
MT-3	1.48	0.30±0.04	-	1.78±0.04	-	0.11±0.02	138	ND
MT-4	1.38	0.41±0.04	111	1.91±0.04	102	0.06±0.03	-	ND
MT-5	1.52	0.33±0.03	-	1.81±0.02	-	0.16±0.05	200	ND

*Data for enzyme activity are showed as mean±standard deviation (SD)

*ND means data not shown and "–" means not increased

*Clearing zone ratio of wild type and mutant strains (MT-1 to MT-5) was investigated on the plate-screening medium, which was incubated at 30 °C for 3 days

*The 3 major enzyme (FPase, CMCase, and β-glucosidase) activities and protein concentrations of wild type and mutant strains (MT-1 to MT-5) were measured using supernatant that was incubated for 5 days in the Mandel's fermentation medium

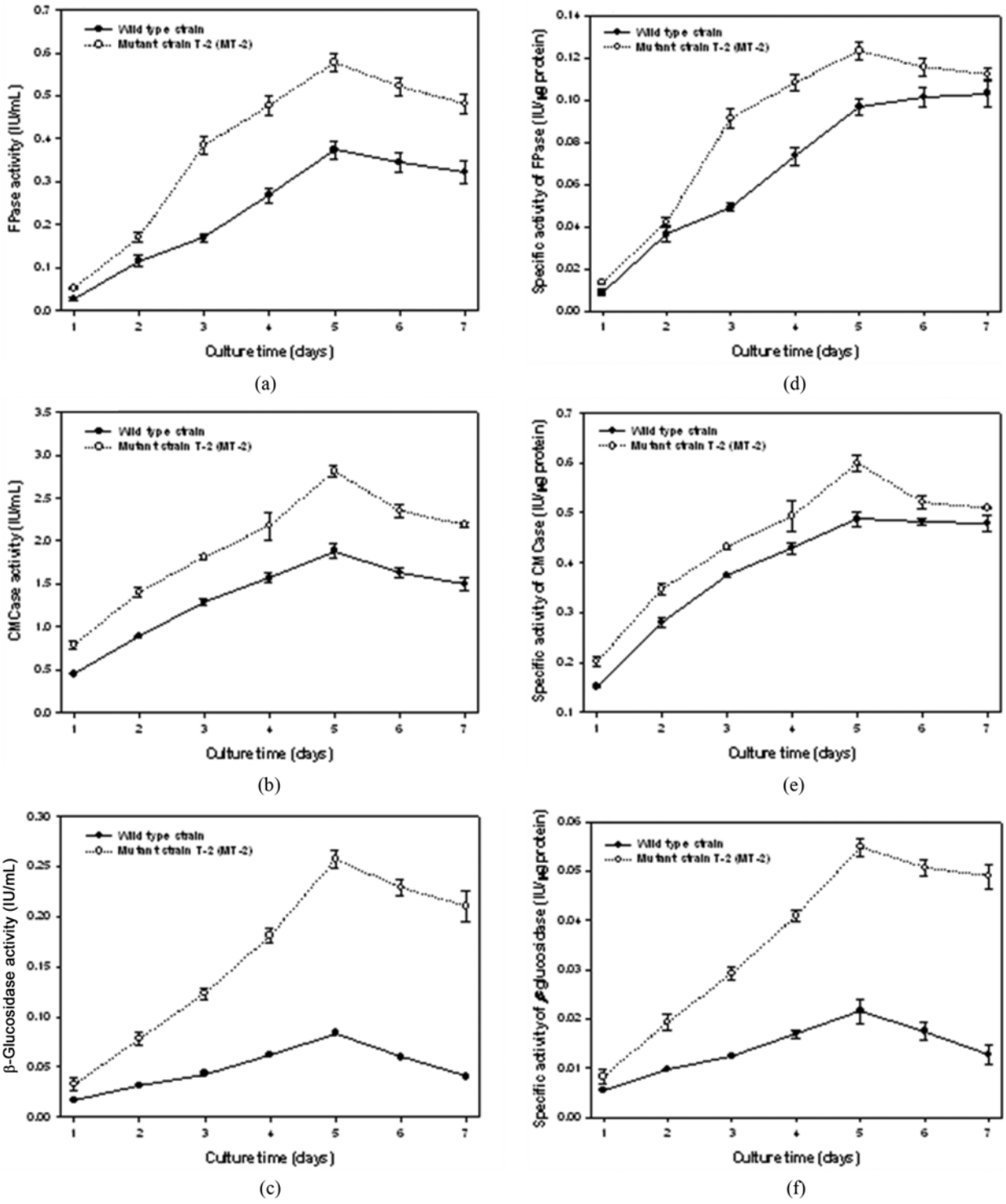

Fig. 3. Growth and production of cellulases and β-glucosidase by wild type and mutant strain T-2 (MT-2). Cultures were grown in Mandels fermentation medium with the addition of 1% microcrystalline cellulose/L, at 30 °C in a shaking incubator.

to the substrate consumption. Another important reason could be the catabolite repression caused by cellobiose accumulation as β-glucosidase productivity is reduced [27]. In addition, the specific activities of three major enzymes produced by MT-2 were also increased concomitant with increasing protein levels, as seen in Fig. 3(d), (e), and (f). Total protein concentration was determined by the Bradford method (Table 1). It was reported that the increased protein production resulting from mutation was correlated with enhanced enzyme production [28]. Notably, the MT-2 produced a high level of β-glucosidase as compared to the other two types of enzymes. According to He et al. [1], it could be assumed that the results with MT-2 may imply a change occurred in the enzymatic properties or production yield of β-glucosidase. Thus, the β-glucosidase was investigated for pH and temperature properties.

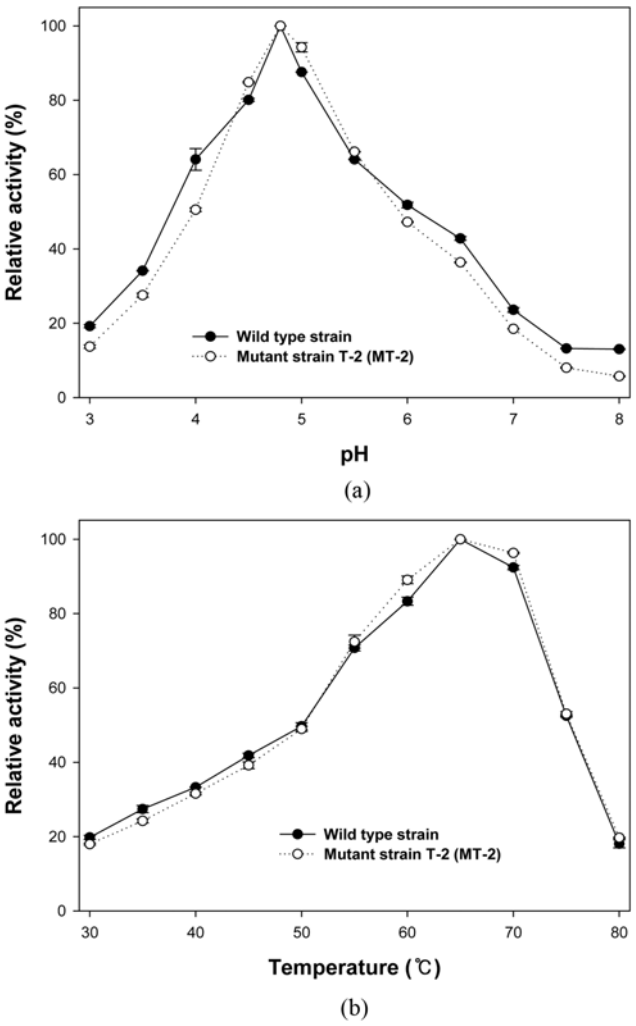

Fig. 4. Characterization of β-glucosidase of wild type and mutant strain T-2 (MT-2) cultured in Mandel's fermentation medium. (a) Optimum pH for the activity of β-glucosidase. pH profile was determined at 50 °C for 20 min using 0.05 M citrate buffer (pH 3.0-6.0) and 0.05 M phosphate buffer (pH 6.0-8.0). (b) Optimum temperature for the activity of β-glucosidase. Temperature profile was determined for 20 min in 0.05 M citrate buffer at pH 4.8, and varying temperatures (reaction temperatures 30-80 °C).

3. Characteristics of β-Glucosidase by Mutant Strain T-2 (MT-2)

To investigate the enzymatic properties or production yield of β-glucosidase, we studied the β-glucosidase excreted by wild type and mutant strain T-2 (MT-2), which were cultured for five days in Mandel's fermentation medium. The optimum pH and temperature of both wild type strain and mutant β-glucosidase were determined to be pH 4.8 and 65 °C, respectively (Figs. 4(a) and (b)). The characteristics of the β-glucosidase produced by MT-2 were similar to those of the wild type strain. However, the β-glucosidase activity by MT-2 was 313% higher than that of wild type strain. These results demonstrate that the production of β-glucosidase was increased by MT-2, but the properties of the enzyme were not changed.

4. Analysis of β-Glucosidase Activity using Zymogram

The components of the cellulolytic systems of the wild type and

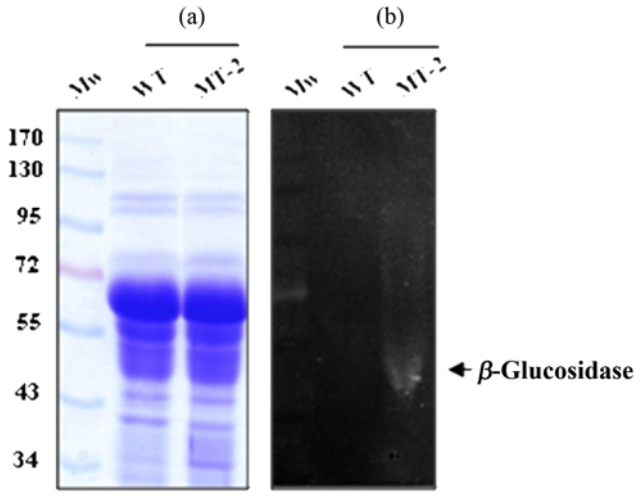

Fig. 5. Identification of β-glucosidase of wild type (WT) and mutant strain T-2 (MT-2) by the MUG-zymogram assay. Proteins separated in SDS-PAGE were from wild type and MT-2, (Lanes 1 and 3) and (Lanes 2 and 4), respectively. Proteins were stained with Coomassie blue R-250 (left panel) and enzyme activities were detected by the zymogram assay (right panel). Mw is molecular weight standard in kDa.

mutant strain T-2 (MT-2) were detected by SDS-PAGE (8% w/v, polyacrylamide gel) performed using the supernatant of a five day culture. As shown in Fig. 5(a), the protein pattern of multi-enzyme complex excreted by the wild type strain and the MT-2 was similar, but the total protein bands of cellulase complex in the MT-2 after proton beam irradiation were much more distinct than those of the wild type strain. The similarity of the cellulase complexes from the wild type and MT-2 suggests that the mutations may not affect the cellulase genes directly, but are perhaps more likely to affect factors that control the synthesis and secretion of the cellulase protein [29]. Therefore, β-glucosidase activity was determined by the MUG-zymogram assay [Fig. 5(b)]. The zymographic analysis of the corresponding band exhibited β-glucosidase activity as fluorescence under UV light, after staining with MUG. A slightly smeared band showing the β-glucosidase activity excreted by MT-2 was detected at 43-55 kDa, but the β-glucosidase excreted by the wild type strain was not observed on the zymogram due to insignificant amounts. These results demonstrate that MT-2 secreted levels of β-glucosidase that were 313% higher than that secreted by the wild type strain. Therefore, the high activity of β-glucosidase produced by mutant strain T-2 (MT-2) enhanced the conversion of cellulose to glucose [30].

CONCLUSIONS

The wild type of *T. reesei* KCTC 6950 was mutated by proton beam irradiation to enhance the activation of enzymes (Fpase, CMCase and β-glucosidase). Most of the cellulolytic strains of *Trichoderma* described in the literature are deficient in the production of β-glucosidase. The absence of β-glucosidase represents a large hurdle in the commercialization of converting cellulose to glucose and other soluble fermentable sugars for the production of ethanol and other products. In this study, a mutant strain T-2 (MT-2) was developed and demonstrated a high production of β-glucosidase, which can lower

the limitations produced by end-products with the two other kinds of enzymes. Therefore, the use of β-glucosidase produced by MT-2 could be advantageous for improving the efficiency of cellulosic bioconversion during bioethanol production.

ACKNOWLEDGEMENTS

This research was supported by the Technology Development Program (309016-5) for Agriculture and Forestry, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea and Midcareer Researcher Program through an NRF grant funded by the MEST (2010-0027563).

REFERENCES

1. J. He, B. Yu, K. Zhang, X. Ding and D. Chen, *Indian J. Microbiol.*, **49**, 188 (2009).
2. A. Bahia and G. Ali, *Biochem. Eng. J.*, **32**, 191 (2006).
3. C. W. Jin, N. K. Rui, C. Joanne, J. Y. Kai, P. L. Xiao, T. N. Chai and J. N. Aninto, *Korean J. Chem. Eng.*, **27**, 469 (2010).
4. T. Wang, X. Liu, Q. Yu, X. Zhang, Y. Qu, P. Gao and T. Wang, *Biomol. Eng.*, **22**, 89 (2005).
5. C. S. Gong, M. R. Ladisch and G. T. Taso, *Biotechnol. Bioeng.*, **19**, 959 (1977).
6. A. Peyman, S. Noraini, A. H. Aidil and M. Y. Wan, *Biotechnol. Bioprocess Eng.*, **16**, 238 (2011).
7. F. Xu, J. Wang, S. Chen, W. Qin, Z. Yu, H. Zhao, X. Xing and H. Li, *Appl. Biochem. Microbiol.*, **47**, 53 (2011).
8. Y. H. P. Zhang, M. E. Himmel and J. R. Mielenz, *Biotechnol. Adv.*, **24**, 452 (2006).
9. B. Henrissat, H. Driguez, C. Viet and M. Schülein, *Nat. Biotechnol.*, **3**, 781 (1985).
10. M. Gritzali and R. D. Jr. Brown, *Adv. Chem. Ser.*, **191**, 237 (1979).
11. I. Masakazu, P. Jeung-yil and T. Mine, *Appl. Microbiol. Biotechnol.*, **87**, 2059 (2010).
12. Z. Z. Xiao, X. Zhang, D. J. Gregg and J. N. Saddler, *Appl. Biochem. Biotechnol.*, **113**, 1115 (2004).
13. W. J. Chirico and R. D. Jr. Brown, *European J. Biochem.*, **165**, 333 (1987).
14. G. Saunders, A. Allsop and G. Holt *J. Chem. Technol. Biotechnol.*, **32**, 354 (1882).
15. H. J. Kwon, Y. J. Park, Y. B. Yoo, S. Y. Park and W. S. Kong, *J. Microbiol. Biotechnol.*, **17**, 1041 (2007).
16. S. B. Kim, J. S. Kim, J. H. Lee, S. W. Kang, C. h. Park and S. W. Kim, *Appl. Biochem. Biotechnol.*, DOI: 10.1007/s2010-011-9204-4 (2011).
17. M. Mandels, I. Hontz and J. Nystrom, *Biotechnol. Bioeng.*, **16**, 1471 (1974).
18. C. K. Ramesh, S. Richa, D. Hena, D. Som and G. Arvind, *Curr. Microbiol.*, **57**, 503 (2008).
19. M. Mandels and J. Weber, *Adv. Cem. Ser.*, **95**, 391 (1969).
20. T. K. Ghose, *Appl. Chem.*, **59**, 257 (1987).
21. M. M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
22. U. K. Laemmli, *Nature*, **227**, 680 (1970).
23. S. A. Lacks and S. S. Springhorn, *J. Biol. Chem.*, **10**, 7467 (1980).
24. B. Amouri and A. Gargouri, *J. Biochem. Eng.*, **32**, 191 (2006).
25. D. Farshad, D. Jacqueline, M. Iraj, T. Philippe and Z. E. Hamid, *New Biotechnol.*, DOI: 10.1016/j.nbt.2011.02.002 (2011).
26. I. V. Solov'eva, O. N. Okunev, V. V. Vel'kov, A. V. Koshelev, T. V. Bubnova, E. G. Kondrat'eva, A. A. Skomarovskii and A. P. Sinitsyn, *J. Microbiol.*, **74**, 141 (2005).
27. J. Zaldivar, J. Nielsen and L. Olsson, *Appl. Microbiol. Biotechnol.*, **56**, 17 (2001).
28. Q. He, N. Li, X. Chen, Q. Ye, J. Bai, J. Xiong and H. Ying, *Korean J. Chem. Eng.*, **28**, 544 (2011).
29. A. L. Allen and C. D. Roche, *Biotechnol. Bioeng.*, **33**, 650 (1989).
30. N. J. Gadgil, H. F. Daginawala, T. Chakrabarti and P. Khanna, *Enzyme Microb. Technol.*, **17**, 942 (1995).